SENSITIVE SENSING BASED ON OPTICAL NONLINEAR WAVE MIXING

[0001]   This application claims the benefit U.S. Provisional Application No. 60/443,225 of the same title filed on January 27, 2003, the entire disclosure of which is incorporated herein by reference as part of this application.

Background

[0002]   This application relates to optical sensing of various materials, including chemical and biological substances.

[0003]   Nonlinear four wave mixing is an optical process in an optical medium where three coherent optical waves interact with one another through nonlinear coupling to produce a fourth coherent signal wave. The nonlinearities of the medium, primarily the third-order nonlinear susceptibility of the medium in some implementations, contribute to such nonlinear coupling. The signal wave includes information on optically-excited atoms or molecules present in the medium where the three input optical waves intersect and hence can be collected to extract information about the medium. The strength of the signal wave is associated with the population of atoms or molecules and the spectral characteristics of the signal wave can be analyzed to reveal the structure of the atoms or molecules of interest. The coherent characteristics of the four-wave mixing signal beam have a number of advantages, including a laser-like signal beam, efficient signal collection, excellent spatial resolution, and sub-Doppler spectral resolution. Hence, four-wave mixing has been widely used as a highly sensitive tool in spectroscopic measurements.

[0004]   Various four wave mixing systems may be used for detection of a minute amount of a substance. For example, U.S. Patent No. 5,600,444 to Tong describes devices and techniques for using two-input-beam forward-scattering

degenerate four-wave mixing to achieve ultrasensative
analytical measurements of an analyte. Backward-scattering
degenerate four-wave mixing has also be used for sensitive
laser spectroscopic detection. See, e.g., U.S. Patent No.

5   6,141,094 to Tong.

Summary

[0005]   This application includes techniques for using four
wave mixing in microarrays with sample cells of biological or
chemical materials. Examples of suitable microarrays include

10   but are not limited to DNA microchips and capillary
electrophoresis microarrays.

[0006]   In one implementation, a microarray is provided to
include DNA cells. The microarray is placed in an optical
degenerate four-wave mixing (DFWM) system operating at an

15   optical wavelength within an absorption spectral range of the
DNA cells to generate a DFWM signal in one DNA cell. The DFWM
signal is collected and measured. Next, the mciroarray is
scanned in position to place other DNA cells in the DFWM
system to get respective DFWM signals.

20   [0007]   In another implementation, a microchip is provided
to include metal ions chelated in a compound in a solution. A
capillary electrophoresis process is performed to separate the
metal ions from the compound in the solution. Optical beams
are directed to overlap on the microchip in a four wave mixing

25   configuration to obtain a wave mixing signal. The wave mixing
signal is then used to determine a concentration of the metal
ions.

[0008]   Laser wave mixing systems are also described in this
application. In one implementation, a system includes a laser

30   to produce a laser beam, means for splitting the laser beam
into a pump beam and a probe beam, means for directing the
pump and probe beams to overlap at a sample location in a
forward-scattering four wave mixing configuration, a
microarray having cells located to place one cell in the

generate a wave mixing signal, and an optical detector located to receive the wave mixing signal.

[0009]   In the above and other implementations, forward-scattering four wave mixing configurations may be used. Backward-scattering four wave mixing configurations may also be used.

[0010]   These and other implementations and their variations are now described in greater detail in the following drawings, detailed description, and the claims.

Brief Description of the Drawings

[0011]   FIG. 1a schematically shows one example 100 of a four-wave-mixing spectroscopic analyzer in a backward-scattering degenerate four wave mixing configuration.

[0012]   FIG. 1b shows one implementation of alignment templates for the system in FIG. 1a, where numerals used are applicable to FIGS. 1a and 1b only.

[0013]   FIGS. 2a, 2b, 2c, and 2d show examples of forward D4WM systems for measuring various materials, where numerals are applicable only to FIGS. 2a through 2e.

[0014]   FIG. 2e shows an example of a backward D4WM system with fiber cables for routing beams.

[0015]   FIG. 3 shows an exemplary instrument that combines the D4WM with a microarray for detecting chemical or biological materials in the microarray cells.

Detailed Description

[0016]   Optical sensing devices and techniques of this application are designed for highly sensitive, selective and high-resolution sensing of various materials based on nonlinear laser wave mixing. Such sensing devices may be implemented using, among others, microfluidic devices, laboratory-on-a-chip, fiber optics, and capillary cells for potential chemical, biological and environmental applications.

Exemplary implementations of nonlinear wave mixing for
measurements in microarrays are described.

[0017]   Nonlinear optical wave mixing may be implemented in
optical sensing systems with different configurations.

5   Examples of such configurations may be found in U.S. Patent
Nos. 5,600,444 issued February 4, 1997 and 6,141,094 issued
October 31, 2000, the entire disclosures of which are
incorporated herein by reference as part of the specification
of this application. Techniques and features in the above-

10   referenced patents may be used or combined with the techniques
described in this application. Notably, the nonlinear multi-
photon laser wave-mixing optical methods may be implemented in
portable, robust and compact systems for a wide range of
applications, including, but not limited to, biomedical

15   applications. Advantages of such techniques and systems
include high spatial resolution and sensitivity, versatile
applications, ease of operations, convenient procedures, and
less tedious chemical steps, etc. Taking advantage of small
probe volumes available, this method can be easily interfaced

20   and adapted to small, relatively portable, robust,
microfluidic devices.

[0018]   As an example, the nonlinear wave mixing techniques
for sensitive high-resolution detection may be implemented
with high temperature atomizers including graphite discharge

25   plasmas, graphite furnace, inductively coupled plasma, and
flame atomizers with detection sensitivity levels in the sub-
parts-per-quadrillion levels. Applications to liquid-phase
samples can achieve high detection sensitivity levels.

[0019]   The laser wave mixing methods offer many potential

30   applications in many fields including chemistry, biology, and
medicine. For example in biotechnology, laser wave mixing
could be used for detecting biomolecules (e.g., proteins,
DNAs, etc.) with or without labels or tags, for studying
enzyme activities, for monitoring smaller chemical/biological

changes more dramatically with less tedious procedures, for
studying bio molecular structures, for analysis of small bio
cells with high spatial resolution, for sensitive detection as
sensors, and many other potential applications.

5   [0020]   The laser wave mixing based detection methods may be
useful for various applications in a wide range of fields for
measuring atoms, isotopes (gas-phase) and molecules (liquid-
phase) at detection levels that may be difficult to achieve
with other sensing techniques. For example, laser wave mixing
10   may be used to improve the detection sensitivity by a factor
of about 1,000 to 1,000,000 relative to other sensing
techniques. For example, preliminary detection limits for
laser wave mixing may be obtained at sub-parts-per-quadrillion
level, sub-attogram, sub-zeptomole, and sub-femto molar
15   detection limits. Laser wave mixing may be effectively
interfaced to popular gas-phase atomizers and liquid-phase
flow systems for highly sensitive detection of e.g., gas-phase
atoms and isotopes, at sub-Doppler spectral resolution and
sensitive detection of liquid samples.

20   [0021]   Laser wave mixing can produce positive signals
against dark background for easy detection with a high
sensitivity. Nanoliter- and picoliter-level probe volumes and
spatial resolution can be achieved. Efficient use of short
absorption path lengths can be achieved in laser wave mixing
25   in various configurations, including simple two-input-beam
planar (2-D) and three-input-beam non-planar (3-D) optical
configurations. As described below, aperture templates may be
implemented in laser wave mixing for effective and reliable
optical alignment. Laser wave mixing allows for relatively
30   low operating laser power, e.g., milliwatts for CW lasers, and
nanojoules for pulsed lasers. Hence, low-power, compact, and
inexpensive solid-state lasers may be used. The nature of the
laser wave mixing allows for effective use of polarization,
wavelength and other modulation methods to enhance the

measurement sensitivity. Optical phase conjugation may be
used in the laser wave mixing to achieve unique optical
measurement advantages not readily available in other optical
methods. Laser wave mixing may be applicable to fluorescing,
5   weakly fluorescing, and non-fluorescing samples.

[0022]   Under proper designs, laser wave mixing may be
easily interfaced with a wide range of chemical instruments.
Examples of the instruments include but are not limited to gas
chromatographs (GC), liquid chromatographs (LC), mass
10   spectrometers (MS), GC-MS, LC-MS, inductively coupled plasmas
(ICP), ICP-MS, high performance/power capillary
electrophoresis (HPCE) systems, flow injection analysis (FIA)
systems.

[0023]   FIG. 1a schematically shows one embodiment 100 of a
15   four-wave-mixing gas-phase spectroscopic analyzer in a
backward-scattering degenerate four wave mixing configuration
where three input beams, i.e., a forward pump, a backward
pump, and a probe, are coupled into the sample under
measurement. A laser 102, e.g., a diode laser, produces a
20   tunable coherent beam 103. The laser 102 is tuned to a desired
wavelength in resonance with a selected spectral line of a
sample under measurement. A mirror 104 and a lens 106 guide
the beam 103 to beam splitters 108 and 110. The beam splitter
108 reflects a portion of the beam 103 as a probe beam 111P
25   and transmits the remaining part of the beam 103 to the beam
splitter 110. The beam splitter 110 produces a forward pump
beam 111F by reflection and a backward pump beam 111B by
transmission. Mirrors 112, 114, and 116 are positioned to
respectively guide the backward pump beam 111B, the probe beam
30   111P, and the forward pump beam 111F so that they cross and
overlap with one another. The probe beam 111P and the forward
pump beam 111F cross each other to form an acute angle. A
sample holder 130, such as a DNA microarray chip or a gas-
phase atomizer (e.g., a graphite furnace electrothermal

atomizer), is used to produce a sample in a location where the
beams overlap. In the following description, an atomizer is
'used as an example for the sample holder 130 to produce a
sample vapor under measurement. The sample holder 130 has a
5   chamber with a top wall 132 and a bottom wall 134, and two
side windows 136 and 138 for receiving optical signals. A
sample is resolved in a solvent which is injected from an
injection port 131 on the top wall 132 into the atomizer 130.
The injected liquid drops to the bottom wall 134 and becomes
10   vaporized. A computer 160 is included in the analyzer 100 to
process and store signals from the photodetector 150. The
computer 160 may also function as a system controller to
control operations of the atomizer 130 or the laser 102.

[0024]   The probe beam 111P, the forward pump beam 111F, and
15   the backward pump beam 111B as shown form a three-dimensional,
non-planar, four-wave-mixing configuration. The probe beam
111P is in a different plane than the plane defined by the
pump beams 111F and 111B. When two input beams intersect at a
small angle in an optical medium, constructive and destructive
20   interferences of these beams form periodic modulation in the
medium. Two of the three input beams write a grating inside
the graphite furnace, and the third input beam scatters off
the grating to produce the fourth coherent signal beam 111S.
The difference between ground- and excited-state populations
25   across the modulation or gratings depends on the excitation
wavelength and the angle between the grating-writing beams.
The signal beam 111S is generated in an optical path different
from the probe beam 111P and can be directly collected by a
photodetector 150 (e.g., a photodiode). A detection module 142
30   is also positioned relative to the furnace chamber 130 to
receive the transmitted probe beam 132 and measures certain
properties of the transmitted probe beam 132. For example, the
detection module 142 may include a wavemeter to measure the
wavelength of the probe beam for spectroscopic analysis.

[0025]   Two alignment templates 120 and 122 are respectively placed on two sides of the furnace chamber 130 for pre-alignment of the three input beams 111P, 111F, and 111B. FIG. 1b shows one exemplary implementation of the alignment templates 120 and 122. Each alignment template may be an opaque mask having at least three specially designed holes for transmitting beams. Thus, templates 120 and 122 also serve as spatial filters to prevent the scattered background light from reaching the detector 150. The templates 120 and 122 may be made by simply forming four small holes, one for each beam involved in a four-wave mixing process, in two thin aluminum plates. The pattern formed by the holes in one template is inversely symmetric with respect to the pattern formed by the holes in another template. In FIG. 1b, the templates 122 and 120 have holes 210A, 220A, 230A, 240A and 210B, 220B, 230B, 240B, respectively. The templates 122 and 120 are positioned relative to the furnace chamber 130 so that holes 210A and 210B define the path of the forward pump 111F, holes 220A and 220B define the path of the probe 111P, holes 230A and 230B define the path of the signal beam 111S, and holes 240A and 240B define the path of the backward pump 111B. The positions of holes 230A and 230B can be determined based on given directions of beams 111F, 111B, and 111P according to the vector conservation requirement of the four-wave mixing process. The pump beams 111F and 111B may be opposing each other, where the beam 111F goes through the holes 210A and 210B and the beam 110B goes through the holes 240B and 240A. When the beams 111F and 111B are counter propagating, they go through the same pair of holes.

[0026]   The positions of the templates 120 and 122 are fixed relative to each other for a desired four-wave mixing configuration. The chamber of the gas-phase atomizer 130 may be adjusted to change its position relative to the templates 120 and 122 so the laser beams can cross and overlap in a

desired position within the chamber. The relative position of a hole relative to other holes in each template is selected so that the four beams 111F, 111B, 111P, and 111S overlap in a common volume with desired crossing angles with respect to one another at a desired spacing between two templates 120 and 122. The common volume is positioned within the furnace chamber 130 at where the sample vapor is located. Preferably, the forward pump beam 111F and the probe beam 111P form an acute crossing angle of less than about 20 degrees, more preferably less than 10 degrees, and most preferably, less than 5 degrees. This crossing angle is important since it affects the sharpness of gratings formed in the sample, the grating periods, and the grating washout due to thermal motion. The dimension of the holes is selected to control the beam size and also determines the volume of the overlap region at a given crossing angle between the beams 111F and 111P. The volume of the beam overlap region is adjustable and is usually smaller than the volume of the sample vapor produced in the furnace 130 so that the entire beam overlap volume is within the vapor volume. The dimension of the holes may be further adjusted to filter out the background noise in the signal beam 111S to achieve a desired signal-to-noise ratio.

[0027]   Once the templates 120 and 122 are placed at their desired positions at both ends of the furnace 130, the optical components then can be pre-aligned. For example, the pre-alignment can be accomplished by using a visible alignment laser beam to produce four alignment beams to respectively trace the paths of beams 111F, 111B, 111P, and 111S. Thus, the first and second alignment templates 120 and 122 can be aligned in their desired positions relative to the chamber 130. Other beam guiding elements and the elements in the path of the signal beam 111S (e.g., detector 150) can also be aligned. Upon completion of the pre-alignment, the actual pump beams 111F and 111B and the probe beam 111P can be generated

to trace the alignment beams. Therefore, implementation of
templates 120 and 122 significantly simplifies the otherwise
time-consuming optical alignment and optical maintenance of
the system. This is especially important when using atomizers
5   that do not fire continuously, and hence, the signal 111S is
not continuously present. Thus, the optical alignment in
general cannot be performed conveniently by using the
generated four-wave mixing signal 111S during the transient
period when the sample vapor exists.

10   [0028]   Referring back to FIG. 1a, the spectrometer 100 may
also include a polarizer 145, an optical bandpass filter 146,
and a lens 148 in the optical path of the signal beam 111S.
These components and the photodetector 150 can be pre-aligned
by using an alignment beam, preferably visible, to trace the
15   path of the signal beam 111S. The optical filter 146 is
configured to transmit light at the signal wavelength and
block light at other wavelengths such as the white light
emitted from the graphite furnace 130. The polarizer 145 is
aligned to have its polarization substantially parallel to the
20   signal polarization to provide polarization discrimination.
The polarizer 145 may also be used to implement polarization
modulated detection techniques to increase the signal-to-noise
ratio in the signal detection. When all three input beams are
vertically polarized, for example, the signal beam 111S is
25   vertically polarized; when one of the three input beams 111F,
111B, and 111P is orthogonally polarized relative to the other
two input beams, the signal beam 111S is orthogonally
polarized. Polarization properties of laser wave mixing can be
advantageously used for many important applications including
30   sensitive circular dichroism spectroscopy. In addition, the
polarizer 145 blocks randomly-polarized background light from
reaching the detector 150.

[0029]   The spectrometer 100 can be operated in either a
continuous sampling mode or a pulsed sampling mode. In the

continuous sampling mode, the laser 102 produces a continuous
wave beam 103. Hence, the signal 111S is also continuous. A
lock-in amplifier 152 and a chopper 154 can be used to
modulate one of the input beams (e.g., the probe beam 111P) in
5   order to increase the signal-to-noise ratio. When the laser
102 is a pulsed laser, analyzer 100 operates in the pulsed
sampling mode and a "boxcar" averager may be used to replace
the lock-in amplifier. In either mode of operation, the total
intensity of the beams 111F, 111B, and 111P is preferably at
10   or close to the saturation intensity of the sample to achieve
an efficient four-wave mixing. The saturation intensity
generally varies from one sample to another depending on
properties of the excited atomic or molecular transition in
the sample. When the total intensity is above the saturation
15   intensity of a sample, the efficiency of the wave mixing
decreases and other adverse effects arise, including power
broadening of the spectral line that reduces the spectral
resolution. In addition, the laser 102 may be configured to
produce a narrow linewidth to achieve a high spectral
20   resolution in the sub-Doppler range since the counter-
propagating configuration of the pump beams 111F and 111B
essentially eliminates Doppler broadening. Further, the
frequency of the laser 102 may be stabilized to avoid mode
hopping and to further improve the spectral resolution and the
25   signal-to-noise ratio. Preferably, the linewidth of the laser
102 is stabilized at about a few MHZ (e.g., 1 MHz).

[0030]   The four-wave-mixing signal in general has a cubic
dependence on the input laser power. The signal strength, and
hence, the detection sensitivity can be further enhanced by
30   using higher input beam intensities up to about the saturation
intensity. The four-wave-mixing signal also has a quadratic
dependence on absorption coefficient. For analytes with low
absorption coefficients, the wave-mixing detection sensitivity
is still comparable to or better than those of conventional

laser methods because of the nonlinear signal properties such as cubic power dependence, virtually 100% optical collection efficiency, and the laser-like coherence properties of the signal beam. For example, unlike laser-induced fluorescence

5   methods where the signal is a small fraction of a widely diffused fluorescence signal laser, the wave-mixing signal is a collimated coherent laser-like beam and hence nearly the entire signal beam can be directed into a photodetector. Furthermore, since wave mixing is an absorption method, both

10   fluorescing and non fluorescing analytes can be measured.

[0031]   FIG. 2a shows one example of a two-input-beam forward-scattering degenerate four-wave mixing F-D4WM arrangement. It should be understood that the particular arrangement of reflectors and beam splitters is provided only

15   as an exemplar only. This system includes an excitation light source 201, which may be a compact diode laser, such as is commonly known. Alternatively, a helium-neon laser, a continuous-wave argon ion laser or a Nd:YAG laser may be used. A laser beam 203 output by the light source 201 is preferably

20   reflected by a reflector 205 and split by a beam splitter 207 to form a first 209 and a second 211 input beam. The second input beam 211 is reflected by a second reflector 213 such that the first 209 and second 211 input beams are preferably generally parallel. The intensity I1, of the first input beam

25   209 with respect to the intensity I2 of the second input beam 211 arriving at the sample cell, I1:I2, is approximately 7:3 to generate the signal in one direction only. The preferred ratio is 1:1 to generate two signal beams.

[0032]   A third reflector 215 and a fourth reflector 217

30   redirect the first input beam 209 and the second input beam 211, respectively, toward a single 100-mm focusing lens 219. The focusing lens 219 preferably focuses and-mixes both input beams 209, 211. A sample holder 221 is placed at the lens' focal point. The sample holder 221 may be a DNA microarray

chip, a sample cell, or other devices holding the sample under measurement. The diameter of both the first input beam spot and the second input beam spot on the sample cell 221 may be approximately 34 micros as one example. The first input beam

5   209 and the second input beam 211 intersect inside the sample cell 221 with an intersect angle which may be approximately 1.5 degrees or less. The small input beam spots allow the system to interface directly with systems in which an analyte is available in a small volume, such as a cell in a microarray

10   chip, the capillary tube of a high power/high performance capillary electrophoresis system (HPCE), the column of a high performance liquid chromatography (HPLC) system, or to directly probe a small volume inside a gas-phase atomizer such as flame, dc plasma, graphite furnace, inductively coupled

15   plasma, with high spatial resolution in diagnostic studies.
     [0033]   In order to optimize the signal strength of a phase-conjugate signal which is generated, the difference in path lengths (or distances traveled) for first input beam 209 and the second input beam 211 are preferably kept to less than the

20   coherence length of the laser. A device for amplitude modulating the second input beam 211, such as a mechanical light chopper 223 (for example, Model 03-OC4000, manufactured and distributed by Photon Technology International Inc., Princeton, N.J.), or any solid state electronic light

25   intensity modulation device, is used. A phase-conjugate signal beam 225 generated in the sample cell 221 is directed to a detector 227, such as a photomultiplier tube (e.g., Model R928, manufactured and distributed by Hamamatsu Corp., Middlesex, N.J.) after passing through a lens 229 preferably

30   having a 250-mm focal length and preferably a filter 231 which in the preferred embodiment is a 514.5 nm laser-line filter when using an argon ion laser. A small aperture 233 is preferably disposed in front of the detector 227 to minimize

background noise due to the scattering of the two input beams
209 and 211.

[0034]   The electrical output signal 235 of the detector 227
is then preferably coupled to a current-to-voltage converter
5   237, the output of which is preferably monitored by a lock-in
amplifier (otherwise known as phase sensitive amplifier) 238
(such as Model 5207, manufactured and distributed by Princeton
Applied Research, Princeton, N.J.). The output from the
detector 227 may also be coupled to other processor components
10   241, such as a strip-chart recorder, personal computer
including an analog to digital converter, or any other such
processing device. Control of the present invention may be
performed by the same computer used to control a HPCE, HPLC,
or atomizer system with which the present invention is being
15   used.

[0035]   In implementations where the sample holder 221 is a
cell, the sample cell 221 may be the capillary of an HPCE
system, the column of an HPLC system, or a gas-phase atomizer
system (e.g., flame, de plasma, ICP plasma, graphite furnace).
20   However, a rectangular glass flow cell with approximately a
0.1-mm optical path length (such as a Type 48, manufactured
and distributed by Starna Cells, Inc., Atascadero, Calif.) may
be used to measure an analyte for other purposes. Naturally,
the sample cell 221 may take any form which can hold a volume
25   of analyte which is at least equal to the spot volume of the
focused input beams 209, 211, and which allows the input beams
209, 211 to enter and the signal beam 225 to exit without
excessive attenuation. Furthermore, the analyte in the sample
cell 221 may be any substance in any phase (i.e., liquid,
30   solid, or gaseous), such as eosin B dissolved in ethanol and
iodine in carbon tetrachloride. The present system is capable
of analyzing solids and gases, as well as liquids.

[0036]   An analyte solution may be delivered to the sample
cell 221 in accordance with the system with which the present

invention is being used. For example, the analyte is delivered
by electrophoresis in an HPCE system or a pump in a HPLC
system. Alternatively, a pump, such as a peristaltic pump 239
may be used to deliver the analyte to the sample cell 221.

5   [0037]   After the two input beams 209, 211 pass through the
sample cell 221, they are blocked by a beam trap 243, and the
signal beam 225 is easily separated and directed toward the
detector 227. An analyte solution with a relatively high
concentration may be used as an "alignment solution" to

10   optimize the optical alignment. A micromolar-level solution
can generate a strong signal that is visible to the naked eye,
thus allowing simple alignment of the present invention.
Signal optimization is performed simply by adjusting the
mirrors and the lenses, and by carefully adjusting the

15   position of the sample cell 221 so that the sample cell 221 is
at the focal point of the wave-mixing lens, while observing
the strength of the visible signal spot on a card (or on a
photodetector for trace-concentration analytes). Of course,
any other means for determining the maximum strength of the

20   signal beam 225 while adjusting the alignment of the system
would be equally acceptable. For example, a self-adjusting
system using feedback from the lock-in amplifier 238 might be
used to determine the optimum alignment of the system. Once
the optical alignment is optimized, the alignment and the

25   signal remain very stable and different analyte solutions
could be flowed through and analyzed without any further
adjustments.

[0038]   FIG. 2b illustrates an implementation two-input-beam
wave mixing system coupled to the capillary tube of a HPCE

30   system. The sample cell 221 is part of the capillary tube of
the HPCE system. One end of the sample cell draws from a
positive pool 249. The other end of the sample cell discharges
into a negatively charged pool 251. A high voltage source is
coupled to, and controlled by, the HPCE system controller 247.

[0039]   FIG. 2c illustrates another two-input-beam system coupled to a column 257 of a HPLC system. The sample cell 221 is coupled at one end to the column 257, and at the other end to a waste pool 259. A pump is coupled to, and controlled by, a HPLC system controller 255.

[0040]   FIG. 2d further shows another example where the laser beams 209, 211, 225 are each transmitted through a fiber optic cable 250. Hence, transmission of laser light through air can be minimized. The fiber optic cable may be prealigned and bound to a substrate to prevent misalignment. The output of the laser source 201 may be coupled directly to the fiber optic cable 250 in known fashion. The fiber optic cable may be split into two sections 253, 255 in known fashion, thus dividing the beam into the first input beam 209 and the second input beam 211. The second section of fiber optic cable 255 may be coupled to an amplitude modulation device 223', such as a well known mechanical chopper, or any solid state electronic light intensity modulation device or an electro-optical modulator. Use of an electronic circuit for modulating the second input beam 211 allows the system to be produced in a compact package. The output of the first section of fiber optic cable 253 and the output from the modulation circuit 223' are preferably coupled to a lens 219. The lens causes the two input beams 209, 211 to be focused to a fine point within a sample cell 221. The input beams 209, 211 are preferably trapped at the opposite side of sample cell 221 by a beam trap 243. A signal beam 225 is generated within the sample cell 221and projects outward through an aperture 233, a lens 229, a line filter 231, and into a photomultiplier tube 227. The path from the sample cell 221 to the photomultiplier tube 227 may, be through a fiber optic cable 228. Alternatively, the path may be through air. In one alternative embodiment of the present invention, two signal beams 225, 303 may be coupled to the photomultiplier tube 227 through a summing lens 259 by

fiber optic cable 261, or each signal beam 225 and 303 may be coupled to a separate photomultiplier or photodiode, via air or fiber optic cables and detected by summing or multiplication.

[0041]   FIG. 2e shows one backward D4WM system having fiber optic cable to minimize alignment difficulties. A fiber optic cable 130 is divided into three 137, 139, 141 such that the signal is split into three beams 107, 109, 117. One input beam 107 is preferably directed to the sample cell 113 at the appropriate angle by the fiber optic cable 139. The second input beam 117 is directed by the fiber optic cable 141 at a modulation device 118 and a beam splitter 123 which passes the entire input beam 117. The input beam 117 is focused by a lens 122. A signal beam 121 is generated within the sample cell 113 and transmitted along the path that the input beam 117 traverses from the beam splitter 123. Upon striking the beam splitter 123, the signal beam 121 is reflected toward an aperture 125. The signal beam 121 can be directed toward the photodetector via fiber optic cable or through air.

[0042]   FIG. 3 shows an exemplary instrument that combines a forward-scattering D4WM sensor with a microarray for detecting chemical or biological materials in the microarray cells. A laser such as an argon ion laser or other suitable laser with the desired laser wavelength is used to produce an input laser beam. The input beam is split into a pump beam and a probe beam via a beam splitter, e.g., with a ratio of 70:30. The pump and probe beams are then recombined and focused on the microarray slide (a microchip, a capillary channel, a waveguide, or a microfluidic device). A template with prearranged holes is placed at the back of the microarray to block all the other beams and only allow the signal beam to pass through to reach a photodetector which receives the signal beam. Other laser wave mixing systems such as the

examples described above and others may also be used in combinations with microarray chips.

[0043]   The combinations of microarray chips with D4WM sensors offer many potential applications in chemical,

5   biological and environmental fields and many advantages over other methods (e.g., fluorescence) including, but not limited to, applicability of both fluorophores and chromophores, better detection sensitivity, better monitoring of small changes in chemical and biological properties (i.e., quadratic

10   signal dependence on concentration), and easier signal collection (virtually 100% collection efficiency).

[0044]   The present techniques may be applied in the a wide range of applications, including but not limited to application of laser wave-mixing spectroscopy for thin-film

15   sensors, fiber optics, laboratory-on-a-chip, capillary cells, sensitive detection of biomolecules with or without labels and tags, sensitive detection of analytes in picomolar, femtomolar and lower concentration levels, sensitive detection of analytes at nanoliter and picoliter-level probe volumes and

20   spatial resolutions (the overlapping volume for the input beams is very small), and sensitive detection of both chromophores and fluorophores. The laser wave-mixing spectroscopy may also be applicable to fluorescing, weakly fluorescing and non-fluorescing samples, and hence, can be

25   used to produce excellent fluorescence-like detection limits for samples that do not fluoresce.

[0045]   In addition, the present laser wave mixing techniques may be implemented in multi-photon laser wave-mixing optical arrangements based on 3-beam, one-laser, simple

30   optical configurations for gas-phase samples; multi-photon laser wave-mixing optical arrangements based on 2-beam, one-laser, simple optical configurations for liquid-phase samples; and template-based optical alignment for the use of two

specially designed templates to provide effective and reliable
forward-scattering optical alignments.

[0046]   With proper designs of the optical configurations
for the laser wave mixing, the laser wave mixing may be easily
5   interfaced with to, among others, various continuously flowing
liquid-phase chemical instruments and gas-phase chemical
instruments. Some examples for the continuously flowing
liquid-phase chemical instruments include high performance
liquid chromatographs, high performance/power capillary
10   electrophoresis systems, flow injection analysis systems and
other similar chemical separation and analytical techniques.
Some examples for gas-phase chemical instruments include mass
spectrometers, gas chromatograph-mass spectrometers (GC-MS),
liquid chromatograph-mass spectrometers (LC-MS), inductively
15   coupled plasma-mass spectrometers (ICP-MS), and other similar
analytical methods.

[0047]   Unique features of the present laser wave mixing
methods allow for the effective use of lasers as compared to
the conventional light bulbs currently used in commercially
20   available analytical instruments. Detection sensitivity levels
may reach orders of magnitude better than currently available
models (1,000 to 1,000,000 times, depending on chemical
properties, experimental parameters, and optoelectronics).

[0048]   The following sections describe several
25   implementation examples to illustrate the features and
advantages of the laser wave mixing in combination with
microarray chips.

EXAMPLE 1: IMAGING OF DNA MICROARRAYS BY LASER WAVE MIXING

30

[0049]   Two-dimensional imaging of a DNA microarray may be
accomplished rapidly using laser wave mixing with the use of
an argon ion laser operating at 515 nm. Intra-spot spatial
resolution is obtained with high reproducibility. Imaging of

a single row of 10 spots is also performed with high
reproducibility. Laser wave-mixing signal is confirmed by
monitoring a nonlinear dependence of signal on laser power
(i.e., a slope of 2.85 is measured as compared to the expected

5 theoretical slope of 3). The lowest concentration of probe
detected that is hybridized with the DNA-microarray is 1 femto
molar (fM) (i.e., 0.8 attogram per microliter or 0.1 molecule
per square micrometer). Research is in progress and more work
is under way to further improve these detection sensitivity

10 levels.

[0050]   Processing procedure (routine steps previously
reported) may be implemented as follows. Suitable DNA
microarrays such those marketed by TeleChem may be used.
Oligonucleotides are attached to a superamine substrate

15 surface, which contains primary amine groups on a glass slide
that carry a positive charge at neutral pH. This charge
permits the formation of ionic bonds with the negatively
charged phosphate backbone of the DNA. A covalent bond is
then formed between the DNA and the surface by ultraviolet

20 light or heat treatment. Chip microarrays are printed with
70-mer oligonucleotides in 200 μm spots and configured as two
identical 10 x 10 sub grids that are spaced 4.5 mm apart.
Each microarray chip purchased is further processed to remove
unbound target sequences prior to hybridization. The chip is

25 subjected to a 2 minute wash at 25 °C in 2 x SSC + 0.1%
sarkosyl solution. This step creates an ionic atmosphere on
the chip so that the printed oligos do not aggregate together.
The chip is then washed for 2 minutes in 2 x SSC solution.
This step removes sarkosyl from the array. Sarkosyl is a

30 detergent that can disrupt future binding reactions. The
microarrays are then treated for 2 minutes at 100 °C in D.I.
H2O and cooled to room temperature. The boiling water
denatures the oligos and helps assure that the oligos are

straight for hybridization. Finally, the microarrays are subjected for 2 minutes in ice-cold 100% ethanol.

[0051]   Hybridization procedure (routine steps previously reported) may be implemented as follows. Microarray chips are hybridized in a hybridization cassette for approximately 4 hours at room temperature. Hybridization takes place under a 22 mm x 22 mm optically flat cover slip with a 5.0 µL probe solution. The probe solution contains 8.0 µL of 1.25 x SuperHyb hybridization buffer and 2.0 µL of the universal probe solution. The universal probe solutions contain the complimentary 9-mer oligonucleotide strands. The 9-mer strands have a Cy3 label on the 5' end. The microarrays are then washed for approximately 1 minute in buffer solutions A, B, and C, which contain 2 x SSC + 0.1% sarkosyl, 2 x SSC and 0.5 x SSC, respectively. These final washing steps break bonds that are formed from the non-specific matching of the probe. The slides are then whipped dried for 2 minutes before 2-dimensional imaging by laser wave mixing.

[0052]   Microarrays are probed, excited, scanned and measured by using a novel forward-scattering degenerate four-wave mixing optical setup based on the absorption of the Cy3 label, which has an absorbance maximum near 535 nm. The 514.5 nm line of an argon ion laser is used as the excitation source for the Cy3 label. The resulting signal is sent through a custom designed precision template and detected by a photodiode. During the spatial scanning process, the laser power used is between 2.5 and 5 mW, but not limited to this range. Microarray spots are scanned with the use of a motorized precision actuator in order to achieve automated and reproducible scans. Background optical noise is determined by scanning the blank glass surface between the spots (i.e., the optical blank). No signal is detected on the blank glass surface, assuring that hybridization and washing are performed effectively. Detection sensitivity is excellent and improving

continuously. Our preliminary results indicate that laser
wave mixing is one of the most sensitive detection techniques
for microarray applications. Laser wave mixing also allows
intra-spot spatial resolution where one can scan, probe and
5 measure bio/chemical contents "within" a single spot on the
microarray. Preliminary intra-spot scanning and probing show
inhomogeniety within each spot due to inhomogeniety in
manufactured preprinted spots. Our laser probe diameter is 25
μm, and hence, it requires much smaller amount of reagents for
10 detection. The microarray chips we purchased come with a
marker spot. The marker spots located in the corners of each
sub-grid contain Cy3-labeled control oligonucleotides. We can
even distinguish variations in signal intensity that are due
to differences in sequence composition of the 70-mer
15 oligonucleotide targets.

[0053]   In this example, the preliminary limit of detection
(LOD) so far for the hybridized probe detected is 1 fM (femto
molar or 1 x 10-15 molar). This is better than many published
results based on other sensing methods. Reproducible results
20 are also obtained when scanning single-spot profiles
repeatedly and when scanning single-row profiles repeatedly.
Laser wave-mixing signal is also confirmed by monitoring a
nonlinear dependence of signal on laser power (i.e., a slope
of 2.85 was measured as compared to the expected theoretical
25 slope of 3).

EXAMPLE 2: IMAGING OF DNA MICROARRAYS BY LASER WAVE MIXING

[0054]   This section describes another example for two-
30 dimensional imaging of a DNA microarray.

[0055]   A DNA microarray consisting of 200 70-mers
oligonucleotide targets was hybridized with a probe solution
containing Cy3-labeled random 9-mers at concentrations ranging
from 10 micromolar to 1 femtomolar. After hybridization, the

DNA microarray was scanned via an argon ion laser operating at
515 nm in a wave-mixing setup. With a laser focused beam
diameter of 32 micrometer, intra-spot resolution was obtained.
The array was moved via a motorized actuator, allowing the

5   scanning of single spots and single rows of the array.
Reproducibility studies were performed. Backward and forward
scans of DNA spot rows and single spots were obtained to
determine the accuracy and reproducibility of the signal and
the movements. Scans of a marker spot were compared with

10   scans of an oligonucleotide sample spot. The lowest
concentration of the probe detected after hybridization with
the microarray was 1 femtomolar with an S/N of 25 for the
marker spot and 7 for the oligonucleotide spot. At S/N of 2,
the limit of detection (LOD) of 2.8e-16 M and 5.4e-16 M were

15   determined for marker and oligonucleotide spots, respectively.
The 1 femtomolar LOD translates to 0.005 fluorophores per μm^2
or between 4 to 5 fluorophores in the probe area. To verify
the signal at the 1 fM concentration, a power study was also
done and the dependency of the signal with laser power was

20   found to be 2.85 with a correlation coefficient of 0.991,
which is close to the theoretical cubic dependence expected
for the method.

Microarray Fabrication

25   **[0056]**   The DNA microarray slides used came from a kit
obtained from TeleChem International Inc (CheckIt Chips,
Sunnyvale, CA, USA) and used as is. The microarray chip (25
mm x 76 mm) is printed with 200 oligonucleotides targets in
200-300 μm spots, and configured as two identical 10x10

30   subgrids spaced 4.5 mm apart. Each spot is about 430 μm from
center to center. The individual oligonucleotide targets
consist of 70-mers from human, mouse and E.coli, which are
printed onto a superamine substrate surface. The superamine
surface contains primary amine groups on a glass slide that

carry a positive charge at neutral pH. This charge permits
the formation of ionic bonds with the negatively charged
phosphate backbone of the DNA. A covalent bond is formed
between the DNA and surface by the treatment with ultraviolet
5   light or heat. There are marker spots placed on each grid
whose location is known to determine if the procedure has
worked.

Hybridization

10   **[0057]**   The hybridization protocols outlined for use with
the kit from Telechem International Inc. were followed with
some minor modifications. Each microarray chip is processed
to remove the unbound target sequences prior to hybridization.
The chip is subjected to a 2 min. wash at room temperature in
15   2X Saline Sodium Citrate (SSC) Buffer and 0.1% sarkosyl
solution. The chip is then washed for 2 min. in 2X SSC
solution. The SSC buffer was made from sodium citrate (Fisher
Scientific), sodium chloride (VWR) and in-house double
distilled water. The 0.1% sarkosyl (sodium lauroyl
20   sarcosinate) solution was made from molecular biology grade
sarkosyl (Fisher Scientific). The microarrays are then
treated for 2 min. at 100°C in distilled water and cooled to
room temperature. Finally, the microarrays are subjected for 2
min. in ice-cold 100% ethanol and dried by slightly shaking.
25   A 10 μL drop of probe solution is added to the microarray and
a cover slip is gently placed on top to distribute the
solution on the entire grid. Microarray chips are hybridized
in a hybridization chamber for approximately 3 hours at room
temperature. The hybridization chamber consisted of an empty
30   pipette tip container with a damp paper towel at the bottom to
prevent the microarray from drying out during the incubation.
After the hybridization, washing of the microarray was
performed with the SSC buffers and drying by gently shaking
was also done.

Probe Solution Dilutions

[0058]   The probe solution consists of a universal random 9-
mer labeled with Cy3 label on the 5' end which will hybridize
5   to all of the spots in the microarray. The probe solutions
were diluted to the desired concentration by the following
serial dilution scheme developed in conjunction with the
manufacturer. In house double distilled deionized water was
used when necessary.

10   [0059]   All solutions were performed fresh each day and
starting from the probe stock solution included in the kit.
The pipette used for the serial dilutions was a 2 μL P-2
Pipetman from Rainin Instruments and it has an accuracy of
1.5% +/- 0.03 μL.

15   [0060]   After hybridization, the microarrays are washed and
then dried and are ready for scanning. Buffer background
noise was determined by hybridizing a microarray slide with
only buffer and no probe solution. No signal was detected on
the glass surface between spots assuring that no hybridization
20   occurred in the glass.

Wave-Mixing Setup

[0061]   Microarrays are scanned in a forward wave-mixing
setup and detected by the absorption of the Cy3 label in the
25   array spots that hybridized. The Cy3 label has an absorbance
maximum near 535 nm. An argon ion laser (Coherent Innova 90)
was used to excite the Cy3 at 515 nm. The argon laser beam is
split by a 70:30 beam splitter and later focused by a 10 cm
focusing lens to the microarray. As the two pump beams get
30   focused on the DNA microarray spot an interference grating is
formed. From this grating, a photon from the probe beam is
diffracted creating a signal beam. The signal is then sent
through a spatial template and detected by a photo diode
detector (ThorLabs, Inc., PDA55, Newton, NJ, USA). A chopper

(Stanford Research Systems, Model SR541, Sunnyvale, CA, USA) connected to a chopper controller (Stanford Research Systems, Model SR540, Sunnyvale, CA, USA) is used on the weaker pump beam. The reference signal from the chopper is sent to a

5 lock-in amplifier (Stanford Research Systems, Model SR810 DSP, Sunnyvale, CA, USA) which was then sent to a desktop computer for collection and analysis. During the scanning process, the laser power ranged from 2 to 4 mW. The microarray was mechanically moved by the use of a motorized actuator (Zaber

10 Technologies, ZLA-28, Vancouver, BC, Canada) controlled via a computer through a Basic program. Glass background noise was determined by scanning the glass surface between the spots. Buffer background noise was determined by scanning the buffer slide and comparing to a probe hybridized slide.

15 **[0062]** Our focused beam diameter is 32 μm and the period in our grating is 13 μm resulting with approximate 2 to 3 grating periods in our probe volume. This formula is an approximation, but it can be useful when trying to get an idea of the approximate number of grating periods present.

20

Resolution Studies

[0063] Our laser spot size was 32 μm at the focal point. This dimension is smaller than the DNA microarray spot of 200-300 μm. The smaller laser spot size allowed us to scan within

25 each spot, and hence, enabling intra-spot resolution. Two different kinds of spots were scanned and their results compared. The first intra-spot scan was of spot 1, which consists of a marker spot. For control purposes, microarray chips have "marker" spots located in the corners of each

30 subgrid. The marker spots contain prelabeled Cy3 single stranded control oligonucleotides. The other type of spot scanned was spot 109, which is a regular oligonucleotide spot. Spots should ideally have a Gaussian distribution of the Cy3 label. This would allow easier quantification of the signal

and less background errors. Instead of the spot having one peak, it has two peaks. This could be the result of the printing process of the target oligonucleotides or the uneven hybridization of the probe. The sample spot does have one

5   maximum peak, but it is not centered. Comparison of forward and backward scans within the same spot was done at 20 μm steps. This was done to determine the accuracy of movement of the actuator used. There can be a small discrepancy between the maximum of the two peaks. This can be attributed to the

10   uncertainty of movement of the actuator, which is related to mechanical parameters and can be seen in the specifications that come with the actuator. Each spot was scanned several times in the same direction to determine the reproducibility of the actuator in the same direction. The points have some

15   variation in them that can also results from the actuator uncertainty in movement. Our method of scanning proves to be more reproducible when the scans are compared in the same direction than when compared from different directions.

[0064]   The resolution chosen to scan the microarray spots

20   was 20 μm. This was done as a middle number since the spot diameter was more closely experimentally calculated to be 200 μm and we wanted to have about 10 scans per spot to keep the time of scanning short and to have enough scans to determine reproducibility. The actuator was capable of the much smaller

25   resolution of 0.1 μm, so it was not a limiting factor. By using 20 μm as the incremental steps for the intra-spot resolution studies, there was some overlap between the fragments scanned. This did not present itself as a problem since the scans were only done to determine reproducibility

30   within different scans performed on the same fragments. By using a shorter focusing lens, the laser spot size could be smaller, and thus, the resolution could be improved.

[0065]   Commercial scanners take an average or blend of incremental signals to obtain spot profiles. This is needed to

obtain clear strong and defined spot signals. As resolution
approaches the spot diameter, the spots appear grainy and
pixilated, and quantification of the microarray data is
impaired. Our method does not suffer from this problem. The
5   signal from the spots is strong when compared to the
background and no multi-scanning of the same spot is really
needed. Also, the laser beams are focused to a small size
diameter which enables the analysis of smaller spots. This
would save money in that fewer reagents would be needed, and
10 higher density arrays could be created with smaller spot
sizes. Since the amount of DNA to spot or the amount of probe
available is often restricted, smaller spots are needed to get
a more accurate DNA microarray analysis results. Methods that
produce sensitive results with these smaller spots are also
15 needed.

Reproducibility Studies

[0066]   We performed rapid automated scans of a row of tens
spots to demonstrate the use of our method in a more realistic
20 automated way. The increments programmed to the actuator were
350 μm. Data is normalized against the maximum signal of spot
100, which is a "marker" spot. The "marker" spot gives off
the highest signal intensity. Spots 10-90 have differences in
sequence composition of the 70-mer oligonucleotide targets
25 varying signal intensity compared to the marker spot. This
experiment was scanned six times demonstrating mechanical and
optical reproducibility.

Sensitivity Studies

30 [0067]   To determine the sensitivity of our method,
hybridizations with different concentrations of probe
solutions were done. The concentration of the probe solutions
used to hybridize the microarray ranged from 1 fM (femtomolar)
to 10 μM. For comparison purposes, signal detection was done

on a marker spot, in this case spot 100, and on a regular
spot, spot 190. Spot 100 was hybridized with 1 fM probe
solution. To determine the background signal from the
microarray that is independent of the probe solution, a

5   microarray slide hybridized with just buffer was detected.
This 'buffer slide' was used to quantify the background of
spot 100 and spot 190 for the LOD studies.

[0068]   Spot 100 on the "buffer" slide and spot 100 on the
1fM slide were analyzed and signal was recorded three times by

10   manually blocking the probe/pump beam. Recording signal three
times shows reproducibility in the wave-mixing technique.
Laser power was 2 mW. The slide with 1 fM probe solution
showed drastically higher signal intensity compared to signal
of the buffer slide. The S/N was calculated to be 25. This

15   translated into a detection limit (S/N 2) of 2.8e-16 M for the
probe solution.

Regular Spot

[0069]   Spot 190 was compared with signal of the same spot

20   on another slide hybridized with no probe solution and
compared to signal obtained from the glass surface between
spots 190-200. A detection limit (S/N 2) of 5.4e-16 M is
determined. Laser power used was 2 mW. The marker spot 100
gave peaks with higher signal intensity than spot 190, as

25   expected.

Effective Hybridized Spot

[0070]   When working with the fM probe hybridized array, it
was thought that not all of the microarray spot was actually

30   hybridized since the probe available is so minute. Studies
were performed to map the actual area that is hybridized on
the array. It was found that there is an effective area of
where the probe solution binds to the target molecules.
Through mapping of the signal generated, it was found that the

effective hybridized area had a diameter of about 84 µm. This
number was calculated from the averages of three spot
profiles. Spot 109, 1 and 160 were mapped alongside the X-
direction using the motorized actuator. It was found that the

5   probe did not bind evenly over the entire spot area. The
probe would bind in selected areas effectively reducing the
hybridized spot area.

[0071]   To our knowledge, having 0.005 fluorophores per μm^2
or between 4 to 5 fluorophores present in the probe area is

10   the smallest detection limit reported so far for this type of
application. For this calculation, it is assumed that the
binding ratio between the probe and the target is one to one.
We know that there is one Cy3 per 9-mer probe strand, since
the synthesis dictated this. According to correspondence with

15   the manufacturer of the chips, the typical DNA hybridization
is one to one, but since the probe is a very short chain it is
possible that it could hybridize in a higher ratio than the
one to one expected, therefore, the ratio could be one to two
or even higher; although the higher probability goes with the

20   lower ratios since the concentration of probe (1 fM) is very
low.

Wave-Mixing Signal Verification

[0072]   To make sure the signal obtained from the microarray

25   was real wave-mixing signal, a power dependency study was
done. A power plot (wave-mixing signal vs. laser power) was
collected from the 1 fM probe solution hybridized array, which
is the lowest one hybridized and the microarray that gives our
LOD. A fitted 2.85 dependence on laser power was achieved.

30   The R^2 value was calculated to be 0.991. The 2.85 result is
very close to the theoretical value of three expected. This
study confirms that our wave-mixing signal is real.

[0073]   Laser wave-mixing offers many advantages as an ultra
sensitive detection method for microarrays. Excellent

sensitivity is achieved by our cubic dependence on laser power and quadratic dependence on signal concentration. This method of detection offers many desirable features including higher sensitivity compared to conventional optical detection

5   methods. To our knowledge, wave-mixing detection of DNA microarrays is the most sensitive detection technique for microarray analysis to date. Other advantages of wave-mixing detection include excellent optical signal collection efficiency because signal is collected against a dark

10   background and the signal is in the form of a coherent laser beam. The small diameter of our laser spot allows for detection of smaller DNA microarray spots which would allow the analysis of higher density array with smaller spot sizes. This will allow the use of fewer reagents and less analysis

15   time. Another advantage of this method is the capability of detecting not just fluorophores, but chromophores as well. This opens the door to more molecules for labeling than the ones currently used, which may have better properties useful in microarray analysis.

20

EXAMPLE 3: SEPARATION AND DETECTION OF Ni AND Cu BY MICROCHIP-BASED CAPILLARY ELECTROPHORESIS USING LASER WAVE MIXING

[0074]   Laser wave mixing, an unusually sensitive optical
25   absorption based method, may be used to detect Cu and Ni (the technique is not limited to these metals) after they are separated by a capillary electrophoresis (CE) system embedded on a microchip. The metal ions are chelated with 4-(2-Pyridylazo) resorcinol (PAR) prior to separation. Wavelength
30   shift by PAR allows optical detection using the 514.5 nm line of an argon ion laser. The lowest concentration detected are 0.26 parts-per-billion and 16 parts-per-billion for Ni and Cu, respectively. This corresponds to excellent preliminary detection limits (S/N = 2) of 75 parts-per-trillion and 864

parts-per-trillion for Ni and Cu, respectively, using this
compact microchip-based chemical separation/analysis system.

[0075]   A forward-scattering degenerate four-wave mixing
optical setup is interfaced to a microchip with built-in
5   microchannels for capillary electrophoresis separation and
detection.   The 514.5 nm line of an argon ion laser is used
as the excitation source with a laser power of 50 mW. A
photodiode is used to monitor the signal beam resulting from
the microchannel on the chip. Four platinum wires inserted in
10   the access holes of the microchip serve as electrodes for the
capillary electrophoresis system. The CE-microchip, obtained
from Micralyne, Inc. (Alberta, Canada) is made of Borofloat
glass. The microchip substrate is 95 mm long, 16 mm wide and
2.2 mm thick. The microchip (Twin T type) consists of an
15   offset microchannel cross with 8 mm long side arms for sample
injection, and an 85 mm long separation and detection channel.
The channels are 50 μm wide and 20 μm deep. A 30 kV power
supply (Model PS/MJ30P0400-11, Glassman, New Jersey) is used
to power the microchip-based CE system via platinum
20   electrodes. The PAR agent is used to chelate the metal ions
with +2 charge at a concentration of 1×10^{-3} M in a 10 mM
ammonium phosphate buffer at pH 7.5. The metal ion stock
solution is obtained from Fisher. A Millipore water system
provides the water for sample preparation and sample solutions
25   are filtered with a 0.22 μm syringe filter. The final low-
concentration metal solutions for detection sensitivity
studies are obtained by serial dilution from a stock solution.

[0076]   The PAR buffer solution is filtered before injection
into the microchip. A series of low-concentration metal
30   sample solutions are prepared by serial dilutions. The
microchip is first conditioned with the PAR-buffer solution
and a baseline laser wave-mixing signal is obtained based on
the slight optical absorption at 514 nm. Once the wave-mixing
alignment is optimized based on this baseline signal, a metal

sample solution prepared in the same PAR-buffer solution is injected using an 8 kV injection voltage. The power supply is then switched from the injection mode to the separation mode, using an 8 kV separation power. The PAR-metal complexes are separated as they travel down the separation channel on the microchip since the pKa of the p-phenolic group of PAR is influenced differently by the central metal ion. Copper elutes first after 20 s. and then Ni at 80 s. Hence, chemical separation of this metal pair is very effective with total resolution. Other metals can be separated with excellent resolution using similar parameters, or if necessary, CE voltage levels can be adjusted conveniently to enhance separation resolution. Rinsing and conditioning of the microchip are done using a syringe pump connected to the microchip via a plastic tubing. The plastic tubing is interfaced to the microchip at the microchip access hole via a pipette tip. Rinsing and conditioning are performed before each sample run to flush out any residue and to avoid any contamination. The capillary electrophoresis system used in this setup is a home-built custom-designed system with manual electrode switching between the injection mode and the separation mode with the high-voltage power turned off.

[0077]   For detection sensitivity studies, a 0.26 parts-per-billion (ppb) Ni sample solution is injected using a 57 s. injection time and a 8 kV injection voltage. This particular design of the microchip does not allow a wide range of sample size injected. One must allow sufficient injection time in order to assure that the sample plug is injected into the side-arm injection microchannel. The injection time (about 50 s.) for this microchip-based capillary electrophoresis system is slightly longer than that for a conventional column-based capillary electrophoresis system. The sample mixture is separated under 8 kV of capillary electrophoresis separation power and detected by laser wave mixing 85 seconds later. A

preliminary detection limit (S/N = 2) of 75 parts-per-trillion (ppt) is determined for Ni using this microchip-based laser wave-mixing system. For Cu detection sensitivity studies, a 16 ppb Cu sample solution is injected using a 70-second

5   injection time. The Cu peak is detected 53 seconds later using an 8 kV separation power. A preliminary detection limit (S/N = 2) of 864 parts-per-trillion (ppt) is determined for Cu. Since Ni has a higher absorption at 515 nm as compared to Cu, Ni yields better laser wave-mixing detection sensitivity.

10   [0078]   In the present example, the metal ions were chelated with the chromophore 4-(2-Pyridylazo)resorcinol (PAR) prior to separation which shifted the chromophore's absorption maximum from about 400 nm to about 500 nm. This shift was detected with an argon ion laser operating at 515 nm. The run buffer

15   consisted of 10 mM ammonium phosphate at pH 7.5 with PAR at 1e-3 M concentration. The separation of the two ions was achieved in about 80 s. The Cu ion came out at about 20 s and the Ni ion came out 60 s later. The laser power used in the runs was 50 mW. The injection and separation voltage was 8

20   kV. Detection limit studies were also performed and the injected LOD for Ni and Cu were 0.26 ppb and 16 ppb, respectively. The detection limits (S/N 2) of 75 ppt for Ni and 864 ppt for Cu were determined. This corresponds to concentration detection limits of 1.3e-9 M for Ni and 1.4e-8 M

25   for Cu. The calculated probe volume for this method was 19 pL. Based on the probe volume used, mass detection limits of 25 zeptomole and 266 zeptomole were determined for Ni and Cu, respectively.

[0079]   The following provides additional information about

30   this separation techniques.

Microchip

[0080]   The CE-microchip was obtained from Micralyne Inc. (MC-BF4-TT100, Edmonton, Alberta, Canada). The chip was made

from Borofloat glass. The dimensions of the chip substrate
were 95 mm by 16 mm by 2.2 mm, with channel plate and cover
plate thickness of 1.1 mm each. The chip was of the Twin T
type channel which consists of a 100 μm offset cross channel,

5   an 8 mm injection channel and an 85 mm separation channel.
The cross-section of the channel was 50 μm in diameter by 20
μm in depth. The chip was conditioned with PAR (1e-3M) buffer
before each CE run and cleaned with methanol and then
Millipore water at the end of the runs. Conditioning was

10 possible by using a syringe pump (A-99EM, Razel Scientific
Instruments, Inc., Stamford, CT, USA).

High-Voltage Capillary Electrophoresis system

[0081]   A Glassman high voltage power supply was used

15 (PS/MJ30P0400-11 30 kV, Glassman High Voltage Co., High
Bridge, NJ, USA). The electrodes consisted of 4 platinum
wires that were inserted in the 4 access holes of the chip
reservoirs. The reservoirs consisted of pipette tips cut to
fit into the chip access holes. Once inserted, the electrodes

20 were not moved from the reservoirs. Alligator clips connected
to the power supply were manually switched from the injection
electrodes (sample reservoir and sample waste reservoir) to
the separation electrodes (buffer reservoir and buffer waste
reservoir). Voltage was turned off while the switching took

25 place. Method of injection used was floating load. Voltage
and current were monitored via two voltmeters. The current
was kept below 30 μA since at higher values than this Joule
heating can impair resolution. Separation and injection
voltage for all runs was 8 kV. Injection on the chip was

30 performed from A to B and separation was performed from C to
D.

Wave-Mixing Setup

[0082]   An argon ion laser (Innova 90, Coherent Inc, Santa
Clara, CA, USA) operating at 515 nm was used with a laser
5   power of about 50 mW. A forward wave-mixing setup was used.
The argon ion laser beam was split via a 70:30 beam splitter
and focused on the sample flowing through the CE-microchip
channel through a 10 cm focusing lens. The signal was
collected and directed to a PDA55 photodiode (ThorLabs, Inc.,
10   Newton, NJ, USA). A chopper (Stanford Research Systems, Model
SR541, Sunnyvale, CA, USA) operating at 200 Hz was used for
background noise reduction. The reference signal from the
chopper was sent to a lock-in amplifier (Stanford Research
Systems, Model SR810 DSP, Sunnyvale, CA, USA) which was
15   connected to a desktop computer for collection and analysis.

Solutions

[0083]   PAR [4-(2-Pyridylazo) resorcinol, monosodium salt
hydrate] obtained from Aldrich (St. Louis, MO, USA). PAR was
20   used at a 1e-3 M concentration in a 10 mM ammonium phosphate
buffer at pH 7.5, made from ammonium phosphate dibasic (A-
1167) and ammonium phosphate monobasic (A-1292) obtained from
Sigma (St. Louis, MO, USA). After the PAR buffer was made it
was filtered with a 0.22 um MCE filter obtained from Fisher
25   Scientific (09-719A Fisherbrand, Tustin CA, USA). The metal
ion solutions were made by serial dilution from a 1000 ppm
Nickel (SN70-100) and Copper (SC194-100) reference solutions
obtained from Fisher Scientific. Water used to make the
dilutions has 18 MOhm resistivity (Millipore). The
30   appropriate metal solutions were mixed with the PAR buffer and
then solutions were sonicated for about 5 minutes before being
introduced into the chip for analysis.

[0084]   The metal has a +2 charge that will bind with the
oxygen of one of the hydroxyl groups of the PAR molecule.

Free PAR has an absorption maximum at around 400 nm. When PAR

binds to the metal, the absorption maximum at 400 nm

decreases, and one gets another peak at around 500 nm. The

extinction coefficients for PAR-Cu and PAR-Ni complexes are

5   around 30,000 and 50,000 (1/Mcm). The new peak at around 500

nm was monitored with an argon ion laser operating at 515 nm.

[0085]   Electroosmosis is a prominent mechanism for

controlling fluid flow in microchannels, requiring surface

charges. The surface charge on the channel walls causes a

10   diffuse layer of counter ions (1 nm – 1 µm thick) to form.

When an electric field is applied across a capillary tube

containing a buffer solution, electroosmotic flow occurs, in

which the solvent migrates toward the cathode or anode. During

the experimental runs, the electroosmotic flow was going from

15   the anode to the cathode. This was in the same flow direction

as the PAR-metal complexes.

[0086]   The type of injection used for this work was

floating or gated injection. The sample is injected by

connecting the positive electrode to the sample reservoir and

20   the negative or ground electrode to the sample waste

reservoir. The buffer and buffer waste reservoirs have no

field applied. After injection, a field is applied from the

buffer reservoir, which now becomes the positive electrode, to

the buffer waste reservoir, which mow becomes the negative or

25   ground electrode.

[0087]   Before each CE run, the channels of the chips were

prefilled with PAR buffer at the same concentration that the

PAR-metal complexes were made. This prevented the appearance

of a PAR peak in the separation since there was no change in

30   PAR concentrations during the run. Wave mixing signal with

the PAR solution was obtained and optimized. This was

possible since the PAR mixture had a slight absorption at 515

nm, which was detectable with our setup. Once the alignment

was optimized, injection of a small amount of metal prepared

in the same PAR-Buffer solution was done, followed by
separation, all using 8 kV.

[0088]   Rinsing and conditioning of the microchip were done
using a syringe pump connected to the chip via plastic tubing
5   with an attached pipette tip at one end which was placed
inside the chip access hole. This operation was performed
before the runs were performed and in between them to make
sure that no contaminants would interfere. The CE-system used
was homemade and the wires had to be changed manually from the
10   injection position to the separation position. This manual
change had to be done with the power off for safety reasons.

[0089]   Injection of a mixture of Cu and Ni at a
concentration of 2 ppm was first done. Copper and Nickel PAR
complexes were made and then mixed and put in the sample
15   reservoir by a disposable syringe. All of the other reservoirs
were filled with PAR buffer solution at the same concentration
as the one already in the microchannels. A platinum electrode
was inserted into each of the reservoirs. Cu came out first
during the separation at approximately 20 s. and then followed
20   by Ni at approximately 80 s.

[0090]   Due to the design of the chip, one gets about the
same size of sample plug injected, regardless of the injection
time, as long as enough time is provided to get to the
separation channel.

25   [0091]   Studies were performed to determine the lowest
concentration of solution injected in the reservoirs that
could be detected by this method. The lowest concentration of
Ni solution detected was 0.26 ppb. The injection time was 57
s and the Ni peak came out at around 85 s. The LOD (S/N 2)
30   calculated was 75 ppt. The Cu concentration injected was 16
ppb, with an injection time of 70 s. The peak came out at 53
s. This gave a LOD of 0.9 ppb Cu. Ni has a higher absorption
at 515 nm than Cu, and thus, it gave a better LOD at this
wavelength. These results were compared to current methods

for metal detection performed in microchips, and to the best
of our knowledge, our results show greater sensitivity than
any other method.

[0092]   The probe volume of the chip was 19 pL. This was
calculated based on our focused laser beam diameter of 35 μm.
This probe volume yields a mass LOD of 25 zeptomole for Ni and
266 zeptomole for Cu. The small diameter of the laser spot
allows the use of smaller chip channels for analysis. The
spot can be even made smaller with a smaller focal length lens
in the setup.

[0093]   To enhance limits of detection (LOD), direct loading
can be utilized to enhance the size of the sample plug. To
reduce sample and reagent consumption, smaller microchannels
can be used. Also, to reduce background, lower concentrations
of PAR buffer should be used or none at all, provided the
metal ions still remain chelated by PAR.

[0094]   Laser wave-mixing offers many advantages as a very
sensitive detection method for CE-microchips. Excellent
sensitivity is achieved by our cubic dependence on laser power
and quadratic dependence on signal concentration. Other
advantages of wave mixing detection include excellent optical
signal collection efficiency because signal is collected
against a dark background and the signal is in the form of a
coherent laser beam.

[0095]   The small diameter of our laser spot allows for easy
coupling of wave mixing to even smaller microchip channels.
This will allow the use of fewer reagents, fewer waste, and
faster separations.

[0096]   Another advantage of this method is the capability
of detecting not just fluorophores, but chromophores as well.
This opens the door to more molecules for labeling than the
ones currently used, which may have better properties useful
in microchip analysis. Newer methods of detection are needed
that do not rely on fluorescence properties of the analytes

for detection. Absorption-based wave mixing is a more
universal detection technique applicable for CE-microchip
analysis which would allow broader applications than
fluorescence.

5

EXAMPLE 4: ULTRASENSITIVE DETECTION OF PROTEINS AND ANTIBODIES BY LASER WAVE MIXING USING A CHROMOPHORE LABEL

[0097]   Laser wave mixing is presented as a sensitive

10   optical method for the detection of proteins bound to
Coomassie Brilliant Blue (CBB), a chromophore label, using a
simple low-power He-Ne laser. Proteins are mixed with the CBB
label and incubated at room temperature for 15 minutes. After
incubation, analytes are flowed through a 350 μm i.d.

15   capillary cell and analyzed using nonlinear wave-mixing
optical detection. Signal can be detected after only a few
minutes of incubation time and maximum signal is obtained
after 10 minutes of room temperature incubation. Different
ratios of CBB to protein are used and studied. The best

20   concentration ratios and incubation times vary depending on
the type of molecule bound to CBB. Each case is studied
separately and the optimum conditions are determined. A
preliminary detection limit of 4.7 x 10^{-19} M (i.e., 2.4 x 10^{-22}
·mol or 3.2 x 10^{-17} g/mL) is determined for BSA. A preliminary

25   detection limit of 9.3 x 10^{-14} M (i.e., 3.7 x 10^{-17} mol or 1.4 x
10^{-11} g/mL) is determined for HPV antibody. All solutions were
prepared in aqueous buffer without the addition of organic
modifiers.

30   **Solutions**
[0098]   Coomassie Brilliant Blue (CBB) dye reagent was
prepared according to instructions from Bio-Rad Laboratories
(Hercules, CA, USA) and used for determining concentration of
solubilized protein. A pH 4 Tris HCl buffer solution (0.50 M)

was prepared in deionized double distilled water filtered with
a Whatman 44 ashless filter. Protein standards of Ovalbumin
and Bovine Serum Albumin (BSA) were obtained from Sigma
Chemical (St. Louis, MO). Human Papillomavirus antibody (HPV)

5   was used as received from US Biological (Swampscott, MA, USA).
All protein stock solutions were prepared by dissolving a
weighed amount of protein in pH 4 Tris HCl buffer solutions.
The stock protein solution was then serially diluted down to
working concentrations. All solutions were made fresh each

10   day.

[0099]   For the time studies, a specific amount of CBB dye
reagent was pipetted into a 2 mL plastic vial containing the
protein solution, mixed with a vortexer and incubated at room
temperature before each measurement. Samples were then pumped

15   into a capillary detector cell and were flowed via a
peristaltic pump.

Wave-Mixing Signal Measurement

[00100]   A forward-scattering degenerate four-wave mixing set

20   up was used for the protein assay measurements. A He-Ne laser
(Uniphase, Manteca, CA, USA; Model 1125P) operating at 632.8
nm was used as the excitation light source. The output of the
laser beam is split by a 70/30 beam splitter which formed two
input excitation beams. The reflected beam from the beam

25   splitter has the higher laser intensity, and therefore, it is
used as the forward pump and probe beam. The transmitted beam
is used only as the pump beam. A mechanical chopper is used
to modulate the amplitude of the pump beam at 200 Hz. The
chopper control was interfaced to a lock-in amplifier

30   (Stanford Research Systems, Model SR810 DSP, Sunnyvale, CA,
USA), which was interfaced to a computer for data acquisition
and display. Together these two input excitation beams travel
equal distances before they are focused at the capillary
detector cell creating a small laser probe volume. Two signal

beams are generated and they propagate in the forward
direction. These signal beams resemble the two input
excitation beams. One signal beam is detected using a
photodetector (ThorLabs, Inc., Model PD55, Newton, NJ, USA)

5   after passing through an iris and a focusing lens.

[00101]   The wave-mixing detector cell is a 350 ▯m inner-
diameter capillary cell (Polymicro Technologies Inc., Phoenix,
AZ, USA) interfaced to a peristaltic pump (Rainin, Woburn, MA,
USA) to keep the analyte solution flowing at a speed of 2.5

10   mL/min. Once the excitation beams are aligned, they counter
propagate through the detector cell. The probe beam
intersects the pump beam inside the cell at a very small
angle. The wave-mixing signal may be maximized by adjusting
the alignment to optimize the signal. An alignment dye

15   solution, Nile blue (Aldrich, Milwaukee, WI, USA), is first
injected into the system to generate the wave-mixing signal,
which is visible to the naked eye, and the setup optimized.
This visible signal beam is then conveniently directed to the
photodetector. The optical alignment remains stable all

20   during the experimental run. To verify this, the alignment
dye is reintroduced to the system after all the protein
solutions are analyzed.

[00102]   In acidic environments, the CBB dye molecule exists
as the unprotonated anionic dye species. The CBB protein-

25   binding dye may also exist in the cationic and the neutral
form, although it is the anionic form of CBB that complexes
with the protein of choice. Modifications of the conjugated
system produce the neutral and cationic form of the dye, which
changes the dye species absorbance maximum. The dye binds to

30   the tertiary amine functional group of the protein causing the
shift in the absorption spectrum. The CBB dye does not bind
to small molecular weight molecules so little interference
should be expected.

[00103]   For the CBB with protein solution, the protein used
was BSA at a concentration of 2.4e-7 M in Tris-HCl buffer.
For the solution of CBB with no protein, only Tris-HCl buffer
was added. The solutions were incubated 20 minutes before
5   being read in the UV-Vis spectrophotometer. A shift in the
absorption maximum of the dye from approximately 490 nm to 600
nm is observed due to the binding of the protein to the
anionic dye. The magnitude of the absorption maximum shift at
higher wavelengths is a function of the protein concentration.
10   This protein-caused shift can be monitored at more than the
traditional 595 nm wavelength that the Bradford assay
dictates. The more protein is available to bind to the CBB,
the higher the shift to higher wavelengths. The 633 nm
wavelength is also a good wavelength to monitor in addition to
15   the 595 nm line. This allows one to use a compact inexpensive
He-Ne 633 nm laser to monitor the amount of protein present in
the wave-mixing studies.

[00104]   The ratio of dye to protein plays an important role
in the binding and the reaction, and consecutively, the
20   detection. Enough protein must be present to bind to the CBB
so that an absorption shift can be observed. If too much CBB
is present, this increases the background signal, thus can
mask the wave mixing signal. An exact amount of CBB must be
present so that it binds to all the protein present in
25   solution and it does not create a large background signal.
The wave-mixing signal generated from CBB-BSA is collected in
three signal intervals using a beam blocker. The beam blocker
prevents the signal from passing through the end of the
experimental set up and onto the detector. By manual movement
30   of the beam blocker three signal peaks are collected. The
wave-mixing signal is compared to a "blank" solution, which
consists of pure buffer and CBB dye. Although, the blank
solution has zero amount of protein a small background signal
was still observed. Minimal absorption can be expected

because the CBB dye still has a small absorption peaks in the monitored range without any bound protein. The limit of detection for BSA calculated for S/N 2 was 4.7e-19 M, which corresponds to 3.2 e-17 g/mL or 32 attogram/mL or 2.35e-22 mol

5   (calculated using a 68 KDa M.W. and a 500 μL sample volume). This corresponds to ~142 molecules of protein that are being detected by the wave-mixing method. To our knowledge, this is the best detection limit for protein detection in a similar assay reported to date.

10   [00105]   To prove the broader applicability of this method, antibodies were also detected by wave mixing. Human Papillomavirus antibody (HPV) is related to tumor detection in people and has been correlated with each of the progressive stages of cervical cancer. Detecting HPV at a more sensitive

15   level is very important since it will aid in earlier diagnosis of possible cancer presence in patients. The HPV antibody is mixed with CBB at different ratios and incubation times and binding ratios are optimized. The protein complex formed was made from 75 μL of CBB dye and 400 μL of HPV and incubated for

20   15 minutes. The experiment followed the same guidelines as for CBB-BSA concentration study. The LOD obtained for HPV was 9.3e-14 M (S/N 2). This corresponds to 1.4e-11 g/mL or 3.7e-17 mol (based on 400 μL sample volume).

       [00106]   Incubation time studies were also performed with BSA

25   and HPV. HPV shows a maximum faster than BSA. This is probably due to the larger mass of HPV which can bind more CBB molecules than BSA. If more binding sites are available for CBB, the reaction will be faster. BSA reached a maximum at around 18 minutes, based on the best fit line, and HPV reached

30   it at around 10 minutes. For this time study, 75 μL of CBB were mixed with 400 μL of HPV at a 3.6e-9 M concentration in tris- HCl buffer. The concentration of BSA was maintained at 1.15e-12 M and 800 μL of BSA was mixed with 25 μL of CBB in the same tris-HCl buffer. After the maximum signal is

reached, signal starts to decrease.   This is probably due to
aggregation of the CBB-protein complex occurring which makes
the complex precipitate out of solution, decreasing the signal
intensity.   This study shows how wave mixing can be almost

5   immediately detected.   No long incubation times are required
for sensitive signal collection.   This eliminates total assay
time which is more apparently important when multiple assays
are required.

[00107]   A time study for Ovalbumin was also made.   This

10   protein behaved similarly as BSA and HPV when bound to CBB and
monitored with wave mixing.   A ratio of 100 µL CBB to 800 µL
Ovalbumin was used.   Data was collected similarly as in other
studies.

[00108]   Such low levels of detection for BSA and HPV were

15   possible since wave missing offers many advantages over
conventional laser-based methods including excellent optical
signal collection efficiency since the signal is in the form
of a coherent laser-like beam, square dependence on sample
concentration and cubic dependence on laser power. Our

20   detection sensitivity levels and concentration limit of
detection are much better than those of many other sensitive
methods.   Our detection limits compare favorably to those of
other methods for protein detection.   Our results are a big
improvement over results obtained by laser-induced

25   fluorescence (LIF).   By performing time studies, we can see
that a long incubation time is not needed to obtain sensitive
results.   We have also cut down on the amount of reagents that
are needed to perform protein detection.   This is very
important when samples are limited due to price or to

30   availability.   Another key feature of this study is that the
samples were analyzed in pure buffer without the addition of
an organic modifier to enhance the wave mixing signal.   This
is very important since for biological analysis it is very

desirable to keep everything at physiological conditions as much as possible.

[00109]   Laser wave mixing offers many advantages as an ultra sensitive detection method for both fluorescing and non-

5   fluorescing analytes. Analytes may be labeled with chromophores, not just fluorophores. Excellent sensitivity is achieved by our cubic dependence on laser power and quadratic dependence on signal concentration. In our non-linear wave-mixing detection, absorbance is determined based on a large

10 absolute positive signal against a virtually dark background. We have demonstrated that wave mixing as an ultrasensitive detection of proteins and antibodies. Advantages of wave mixing includes more sensitive detection, more rapid analysis, less amount of reagents used, lower cost and less

15 environmental waste, and hence, wave mixing is an attractive novel method for protein determination.

[00110]   Only a few implementations are disclosed. However, it is understood that variations and enhancements may be made.